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(54) Title: RIBOZYME-MEDIATED CONTROL OF GENE EXPRESSION

(57) Abstract

This invention provides methods for using ribozymes to modify the development of an embryo and to control the expression of an endogenous gene in a vertebrate. In particular, methods for inhibiting the expression of neuregulins, growth/differentiation factor-8, and interferon are disclosed. Ribozymes and vectors useful in these methods are also provided.

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RIBOZYME-MEDIATED CONTROL OF GENE EXPRESSION

BACKGROUND OF THE INVENTION

a) Field of the Invention

The present invention relates to methods for using ribozymes to control gene expression in animals, to ribozymes and ribozyme-encoding vectors useful in these methods, and to transgenic animals modified by the ribozymes.

b) Description of Related Art

Targeted gene disruption is a useful method for the study of gene function, however, the complete disruption of a gene may be problematic. Late-acting effects of a mutant or missing gene on the organism are easily obscured by early effects; often, incomplete development or even premature death of the organism are attributable to the mutation. The ability to disrupt genes with spatial and temporal specificity (conditional gene inactivation) would be highly desirable.

An example of the need for conditional gene inactivation methods can be seen in attempts to study neuregulin function through traditional gene disruption methods. Neuregulins (NRGs) are a set of polypeptide growth factors whose signaling through the ErbB receptors is essential to the growth and differentiation of many cell types in culture (Burden and Yarden, 1997, *Neuron*, 18:847-855). Although studies with NRG mutant mice have demonstrated that these growth factors are also essential regulators of cellular differentiation *in vivo*, the mid-embryonic death of these mutants precludes an analysis of hypothesized neuregulin roles in later aspects of development (Meyer and Birchmeier, 1995, *Nature*, 378:386-390; Lee et al, 1995, *Nature*, 378: 394-398; Gassmann et al., 1995, *Nature*, 378:390-394).

Targeted gene disruption with spatial and temporal specificity is also of interest for generating desired phenotypes among commercially valuable animals such as chickens and cows. For instance, growth / differentiation factor-8 (GDF-8) is a protein that serves as a negative regulator of skeletal muscle growth

(McPherron et al, 1997, *Nature*, 387:83-90; McPherron and Lee, 1997, *Proc. Natl. Acad. Sci. USA*, 74:12457-12461). GDF-8 null mice were found to possess muscles 2-3 times the normal size. Control over GDF-8 gene expression should produce appropriate increases in muscle mass in birds, mammals, and fish.

Ribozymes are likely to be superior to antisense oligonucleotides as agents of gene inactivation for several reasons. Like antisense oligonucleotides, ribozymes are unlikely to induce an immunogenic response. Moreover, ribozymes are capable of multiple turnover. This means that a single ribozyme molecule is able to cleave many molecules of target RNA. This is not the case for antisense oligonucleotides, which suggests that ribozymes are more efficient in achieving the desired effect. Further, complications in antisense strategies relating to the extent of binding of the antisense molecules to the target is not a concern in strategies employing ribozymes because once the ribozyme binds to target mRNA, the mRNA is typically rapidly and irreversibly cleaved. Additionally, antisense molecules do not offer the same degree of target specificity as ribozyme sequences. Single mismatches or base-substitutions near the cleavage site can effectively eliminate catalytic activity of a ribozyme, whereas similar mismatches in antisense molecules do not prevent their action.

Despite the appeal of using ribozymes for gene inactivation, their potential has not been fully realized. Perhaps the main obstacle to the use of antisense and ribozyme-based strategies is the reliable delimitation of accessible target regions within large RNA molecules. These regions must be open to base-pairing reactions that lead to formation of a hybrid "kissing complex" between ribozyme (or antisense reagent) and target (Wagner and Simons, 1994, *Annu. Rev. Microbiol.* 48:713-742). Obstacles arise because large mRNAs invariably exhibit complex secondary and tertiary structures that are characterized by significant intramolecular base pairing (Uhlenbeck et al., 1997, *Cell* 90:833-840).

Currently, three strategies are generally used in an attempt to circumvent the problem of blocked target sites. The first of these is a shot-gun approach. In this

strategy, numerous short oligonucleotides complementary to sequences distributed throughout the target RNA molecule are more or less randomly selected and screened for inhibitory effects - typically diminution of the level of target RNA or its encoded protein, or of the bioactivity of the encoded protein (Wakita and Wands, 1994, *J. Biol. Chem.* 269:14205-14210). A second approach relies on enzymatic digestion. This strategy is based on the use of single-strand, DNA-RNA duplex, and nucleotide-specific RNases to probe the structural features of a target RNA directly (Ehresmann et al., 1987, *Nucl. Acids Res.* 15:9109-9128; McSwiggen, U.S. Patent No. 5,525,468). The third approach which involves the use of thermodynamic modeling has the distinct advantage of only requiring knowledge of the target RNA sequence. In this strategy, most-probable RNA secondary structures are predicted from sequence data and minimum free energy parameters by using a computer program that minimizes the free energy associated with hydrogen-bonded base-pairs, stacked hydrogen bonds, loop structures, etc. within RNA molecules (Jaeger et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:7706-7710; Zuker, 1989, *Science* 244:48-52). The accuracy of thermodynamic modeling, when carried out for transfer and ribosomal RNAs whose structures have been directly and independently solved, is often 80% or better. Since the first two strategies are tedious and labor-intensive, they have not been widely accepted as methods for the design of exogenous antisense molecules. The fidelity and reliability of the application of the third method in a biological context, i.e., in cultured cells or whole animals, has not been systematically examined.

SUMMARY OF THE INVENTION

This invention provides for retroviral expression vectors comprising nucleic acid encoding at least one ribozyme, wherein said ribozyme which specifically cleaves a non-viral mRNA. In one embodiment, the ribozyme comprises a ribozyme-tRNA hybrid.

This invention further provides for viral expression vectors comprising nucleic acid encoding at least one ribozyme-tRNA hybrid, wherein the ribozyme component specifically cleaves a non-viral mRNA and wherein said expression vector is in association with a delivery vehicle selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a lentivirus, a herpes simplex virus, and a vaccinia virus.

In another embodiment, the invention provides for host cells comprising expression vectors, as defined above.

Still another embodiment of the present invention provides for non-human, transgenic animals and embryos comprising cells modified with exogenous nucleic acid which encodes at least one ribozyme, wherein said ribozyme is capable of specifically cleaving mRNA endogenous to said transgenic animal/embryo.

This invention also provides methods of controlling expression of a gene in an animal or embryo and methods of modifying embryonic development utilizing expression vectors encoding at least one ribozyme or a ribozyme-tRNA hybrid. Specifically, the methods comprise the introduction of one of the aforementioned expression vectors into the animal or embryo, transcription of the ribozyme sequence, and cleavage by the transcribed ribozyme of about 5% to about 100% of the mRNA transcripts of the target gene.

This invention also provides for methods of controlling expression of a gene in an animal or embryo utilizing an invention ribozyme. These methods comprise the introduction of ribozyme into the animal or embryo, and cleavage by the ribozyme of about 5% to about 100% of the mRNA transcripts of the targeted gene.

Still other embodiments of the invention provide a ribozyme which specifically cleaves neuregulin mRNA, a ribozyme which specifically cleaves growth/differentiation factor-8 mRNA (GDF-8) and a ribozyme which specifically cleaves interferon mRNA.

Also provided is a method of determining the efficacy of a ribozyme sequence for controlling gene expression by transfecting a cell in vitro with an

expression vector comprising nucleic acid encoding both a ribozyme and an mRNA sequence, wherein said ribozyme cleaves said mRNA sequence and determining the level of mRNA transcripts in the cell relative to the level of mRNA transcripts produced in a cell by a vector encoding only the mRNA target sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of a neuregulin-cleaving ribozyme-tRNA hybrid and four selected target sites in the neuregulin mRNA. Numbers indicate nucleotide positions of cut sites in the chicken neuregulin mRNA.

Figure 2. Neuregulin-cleaving ribozyme delivery constructs. (A) Diagram of neuregulin-ribozyme dual expression plasmid for cell culture assay. (B) Schematic diagram of the replication competent retrovirus used for delivery of ribozyme-tRNA transgenes to developing chick embryos. Retroviruses contained either four independently transcribed ribozyme-tRNAs (as shown), or alternatively, the single ineffective ribozyme-tRNA RZ_{NRG156} as a control.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides retroviral expression vectors comprising a nucleic acid which encodes at least one ribozyme wherein said ribozyme specifically cleaves a non-viral mRNA.

A "ribozyme" is a catalytic RNA molecule. Many ribozymes are known to catalyze the hydrolysis of phosphodiester bonds under physiological conditions. The ribozymes of the present invention catalyze the sequence specific cleavage of a second RNA molecule, preferably an mRNA transcript. In general, ribozymes bind to a target RNA through the target binding portion of the ribozyme which flanks the enzymatic portion of the ribozyme. The enzymatic portion of the ribozyme cleaves the target RNA. Strategic cleavage of a target RNA destroys its ability to directly or indirectly encode protein. After enzymatic cleavage of the target has occurred,

the ribozyme is released from the target and searches for another target where the process is repeated.

In a preferred embodiment of the invention, the ribozyme is a hammerhead ribozyme, a small RNA molecule derived from plant viroids (Symons, 1992, *Ann. Rev. Biochem.* 61: 641-671; Clouet-D'Orval and Uhlenbeck, 1996, RNA, 2:483-491; Haseloff and Gerlach, 1988, *Nature* 334:585-591; Jeffries and Symons, 1989, *Nucleic acids Res.* 17: 1371-1377; Uhlenbeck, 1987, *Nature* 328:596-600).

In other embodiments, the ribozyme may be a group I intron, a hairpin ribozyme, VS RNA, a hepatitis Delta virus ribozyme or an Rnase P-RNA ribozyme (in association with an RNA guide sequence). Examples of hairpin motifs are described by Hampel et al., 1990 *Nucleic Acids Res.* 18:299 and Hampel and Tritz, 1989, *Biochemistry* 28:4929; an example of the hepatitis delta virus motif is described by Perrotta and Been, 1992, *Biochemistry* 31:16; an example of the RNAseP motif (associated with an external guide sequence) is described by Yuan et al., Patent No. 5,624,824; a Neurospora VS RNA ribozyme motif is described in Saville and Collins, 1990 *Cell* 61: 685-696, Saville and Collins, 1991, *Proc. Natl. Acad. Sci. USA* 88: 8826-8830, Collins and Olive, 1993 *Biochemistry* 32: 2795-2799; the group I intron is described in Cech et al., U.S. Patent No. 5,354,855. The above-mentioned motifs should not be considered limiting with respect to the present invention and those skilled in the art will recognize that ribozymes that may be utilized herein comprise a specific substrate binding site which is complementary to the target mRNA. Such ribozymes also comprise an enzymatic portion which imparts RNA cleaving activity to the molecule. The enzymatic portion resides within or surrounds the substrate binding site.

The ribozyme utilized herein may be expressed as a discrete molecule, however, in a preferred embodiment, the ribozyme is part of a ribozyme-tRNA hybrid. Embedding the ribozyme sequence within a tRNA expression cassette (sometimes referred to herein as "ctRNA") has several advantages. For example, the tRNA expression cassette is a small piece of nucleic acid that is non-selectively

transcribed by RNA polymerase III, at constitutive levels in most cells. Moreover, the higher order structure of the tRNA may also protect the ribozyme from degradation by cellular nucleases. In a further preferred embodiment, the ribozyme is positioned in the anticodon loop of the tRNA sequence. In a still further preferred embodiment, the ribozyme sequence replaces the anticodon triplet in the anticodon loop. The anticodon of the tRNA is unstructured and exposed to solution and its replacement is least likely to be disruptive to the rest of the tRNA structure.

In one embodiment, the tRNA sequence that is associated with the ribozyme sequence is a chicken alanine tRNA ($tRNA^{Ala}$) gene sequence. Other transfer RNA sequences may be used, such as those of $tRNA^{Tyr}$, $tRNA^{Met}$, and $tRNA^{Val}$. It is preferable to use a tRNA gene sequence that corresponds to the host that will be transformed with the ribozyme. In each case, transcription of ribozyme-tRNA hybrids is facilitated by RNA polymerase III-based promoter sequences within the tRNA coding sequence.

Essentially any non-viral mRNA sequence may be targeted by ribozymes of the present invention. One of ordinary skill in the art will recognize that the efficiency of the cleavage reaction will vary depending on the target site chosen. One method for choosing target sites is described in Example 1. When this method of selection is used in conjunction with a cell culture assay for testing ribozyme efficacy as in Example 5, the present invention can be effectively utilized to cleave nearly any mRNA sequence *in vivo*. Preferred targets of the present invention are neuregulin mRNA, GDF-8 mRNA, and interferon mRNA.

A "vector" is defined as a polynucleotide comprised of single strand, circular, or supercoiled DNA or RNA. The term "expression vector" as used herein refers to viral vectors. For purposes of the present invention, it is understood that the ribozyme-tRNA sequence is positioned within the expression vector in such a manner as to facilitate transcription of the catalytically active RNA molecule *in vivo*. Integration of the ribozyme-tRNA construct within the host cell genome may

occur but is not required. Alternatively, the ribozyme or ribozyme construct may be delivered in a liposome or via a cationic lipid complex.

Multiple copies of the ribozyme may be positioned in the expression vector. These ribozymes may target the same or different sites in the mRNA. The ribozymes are preferably oriented in a direction opposite to that of the viral genes. In a preferred embodiment, multiple ribozyme-tRNA hybrid sequences, targeting different sites on a single mRNA target, are positioned in tandem within the vector and oriented backward with respect to transcription of viral genes (Fig. 2B). Ideally, the multiple ribozyme-tRNA cassettes arranged in tandem will be designed to cleave all isoforms of a gene in a given tissue.

In one embodiment the retroviral vector is of the RCAS series (replication-competent, avian leukemia virus long terminal repeat, splice acceptor retrovirus vectors) or RCAS(BP) series (RCAS bearing a *pol* gene from the Bryan high-titer strain of rous sarcoma virus). Other possible vectors include RCAN, RCOS, and RCON type vectors (Petropoulos and Hughes, 1991, *Journal of Virology*, 65: 3728-3737; Hughes et al, 1987, *Journal of Virology*, 61:3004-3012). Vectors used in the present invention may be replication-competent or replication-defective, but are preferably replication-defective. Replication-defective vectors derived from the reticuloendotheliosis viral vector (REV-derived) are useful in the administration of the invention ribozyme to avian animals and embryos (MacArthur et al., WO 97/47739).

Other retroviral vectors may be used for administration of the invention ribozyme. The vectors may be derivatives of RCAS or RCAS(BP) that possess an *env* gene appropriate for the desired host. Alternatively, other recombinant retroviral vectors may be used. For example, amphotropic murine leukemia virus (MLV) retroviral vectors have been shown to be useful in gene transfer to porcine cells (Squire et al., 1989, *Am. J. Vet. Res.*, 50:1423-1427). Bovine leukemia virus (BLV)-based vectors are suitable for use in either bovine or ovine cells (Milan et al., 1991, *Journal of Virology*, 65:1938-1945.) Also, amphotropic Moloney murine

leukemia virus (MoMLV)-based recombinant retroviral vectors may be used in the present invention. These vectors have been used in species as diverse as sheep and zebrafish (John, et al., 1994, *J. Leukocyte Biology*, 55:785-792; Lin, et al., 1994, *Science*, 265:666-669).

This invention further provides a viral expression vector comprising nucleic acid encoding at least one ribozyme-tRNA hybrid, wherein the ribozyme component of the hybrid is capable of specifically cleaving non-viral mRNA and wherein said expression vector is associated with a delivery vehicle selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a lentivirus, a herpes simplex virus, and a vaccinia virus. Some of the viral vectors known to those skilled in the art are reviewed in Ali et al., 1994, *Gene Therapy*, 1:367-384 and Smith, 1995, *Annu. Rev. Microbiol.*, 49:807-838. Recombinant ovine adenoviruses useful in the present invention can be found in Xu et al., 1997, *Virology*, 230: 62-71. Recombinant bovine herpesvirus-1 vectors useful in the present invention are disclosed in Raggo et al, 1996, *Virology*, 221:78-86.

Another embodiment of the invention provides for host cells comprising a viral expression vector that encodes a ribozyme-tRNA hybrid capable of specifically cleaving a non-viral mRNA sequence.

Still another embodiment of the present invention provides for transgenic animals or embryos comprising one or more cells modified with an exogenous nucleic acid encoding at least one ribozyme, wherein said ribozyme is capable of specifically cleaving an mRNA target endogenous to said transgenic animal or embryo. The animal or embryo may be avian, murine, porcine, bovine, ovine, or piscine. In a preferred embodiment, the animal is a chicken or turkey, or embryos thereof.

The term "exogenous" as it relates to nucleic acids, denotes nucleic acids which are not native to the host or cell, or which are present in the host in other than its native environment (e.g. as part of a genetically engineered DNA construct). The term "endogenous" refers to a nucleic acid originating or produced

within the host or one of its parts. Mutated, recombined, or viral genes are not considered endogenous for purposes of this invention.

This invention also provides methods of controlling gene expression in animals and embryos, methods of controlling gene expression in embryos, and methods of modifying embryonic development. These methods comprise the introduction of one of the aforementioned expression vectors into the animal or embryo, transcription of the ribozyme sequence contained in the vector, and cleavage by the transcribed ribozyme of about 5% to about 100% of the mRNA transcripts of the target gene.

The desired degree of inhibition of gene expression will vary depending upon the gene that is targeted. In order to test for gene function, for instance, it is preferable to cleave greater than about 50% of the mRNA transcripts, preferably from about 80% to about 100% of the mRNA transcripts. In some cases, however, only moderate inhibition of gene expression is desired, such as where inhibition of gene expression leads to a desired phenotype in a commercially valuable animal. In such cases, cleavage of a smaller fraction (about 25% to 50%) of mRNA transcripts may be preferred.

Several methods may be employed to achieve control over the percentage of mRNA transcripts cleaved. A decrease in the amount of mRNA cleavage can be achieved by employing less catalytically active ribozyme, incorporating fewer copies of the ribozyme in the expression vector, or targeting less accessible sites on the mRNA target. The reverse is also true. Manipulation of the promoter sequences in the viral vector can also be useful in establishing control over the extent of mRNA cleavage. A strong promoter (such as a pol III promoter) may be used for transcription of the ribozyme sequence if a high level of cleavage is desired; a weaker promoter (such as a pol II promoter) may be used if fewer transcripts and less cleavage is desired. Inducible regulatory elements may also be introduced into the vector to fine-tune control of expression. All the parameters

which affect the percentage of cleaved mRNA transcripts may be systematically tested using the cell culture test discussed below and in Example 5.

The methods of controlling, or inhibiting, or modifying gene expression disclosed herein are applicable to avian, murine, porcine, bovine, ovine, and piscine animals and embryos as well as other vertebrate animals and embryos, such as humans.

Still another embodiment of the invention provides for ribozymes which specifically cleave neuregulin mRNA. Ribozymes which specifically cleave growth/differentiation factor-8 mRNA and ribozymes which specifically cleave interferon mRNA are also provided by the present invention.

In a preferred embodiment, a neuregulin-cleaving ribozyme has one of the following sequences:

RZ_{NRG89} (SEQ ID NO:1):

5'-GTAACTCTGTTCTGATGAGTCCGAGAGGACGAAACGTCCGTCTGAGCTCTGCA-3'

RZ_{NRG156} (SEQ ID NO:2):

5'-GCACTAGCTTCTCTGATGAGTCCGAGAGGACGAAACCCACAGCAAGAGCTCTGCA-3'

RZ_{NRG438} (SEQ ID NO:3):

5'-GTTGTGAGATCTGATGAGTCCGAGAGGACGAAACTTGTCCCAGAGCTCTGCA-3'

RZ_{NRG637} (SEQ ID NO:4):

5'-GCCGTTTCTGGCTGATGAGTCCGAGAGGACGAAACAGTTCCTCGAGCTGCA-3'

In a further preferred embodiment, the neuregulin-cleaving ribozyme cleaves the chicken neuregulin mRNA between bases 89 and 90, between bases 156 and 157, between bases 438 and 439, or between bases 637 and 638. In each of these cases, the cleavage site is preceded by the triplet sequence GUC.

The ribozyme sequences may be obtained by transcription (either *in vitro* or *in vivo*) of appropriate DNA sequences operably linked to RNA polymerase promoter, such as the T7 RNA polymerase or SP6 RNA polymerase promoter. Alternatively, synthetic ribozymes may be prepared and utilized herein. Methods for chemical synthesis of RNA are described in Usman et al., 1987, *J. Am. Chem. Soc.*, 109:7845-7854 and in Scaringe et al., 1990, *Nucleic Acids Res.*, 18:5433-5441. Such methods utilize common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. Other methods are known to those skilled in the art. Modification of the RNA of the ribozyme may be used to render the ribozyme resistant to rnases and bases or to help facilitate delivery by diffusion across the cell membrane (by partially counteracting the negative charge of the polyphosphate backbone of the RNA). Possible modifications include, but are not limited to, the use of 2'-O-methyl groups, 2'-amino groups, and phosphorthioates.

The invention ribozymes may have a structural motif characteristic of a hammerhead ribozyme, a group I intron, a hairpin ribozyme, VS RNA the hepatitis Delta virus, or RnaseP RNA (in association with an RNA guide sequence) as discussed above. The ribozymes have at least 14 contiguous nucleotides complementary to the target mRNA. In a preferred embodiment, the ribozyme is a hammerhead ribozyme which comprises between about 14 and about 28 base pairs complementary to the targeted mRNA. In a further preferred embodiment, the

ribozyme comprises between about 18 and about 24 base pairs complementary to the targeted mRNA.

Host cells containing ribozymes which specifically cleave neuregulin mRNA, growth/differentiation factor-8, and interferon mRNA are also encompassed by the present invention.

The invention further provides a method of controlling gene expression in an animal or embryo with a ribozyme which specifically cleaves neuregulin mRNA, growth/differentiation factor-8 mRNA, or interferon mRNA. The method comprises introduction of the ribozyme into the animal or embryo, resulting in cleavage of about 5% to about 100% of the mRNA transcripts of the target gene. The ribozyme can be introduced into the animal or embryo directly, or alternatively, nucleic acid encoding the invention ribozyme can be introduced. Methods useful for exogenous delivery of ribozymes to a situs are described in Thompson et al., U.S. Patent No. 5,599,704, herein incorporated by reference. The anti-neuregulin, anti-interferon, or anti-GDF-8 ribozymes of the present invention may also be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to cells in a non-viral manner.

The present invention also provides a cell culture method useful in determining the efficacy of a ribozyme for controlling gene expression. This method first involves transfecting a cell *in vitro* with a dual- expression vector that comprises nucleic acid encoding both an mRNA and a ribozyme designed to cleave the mRNA sequence on a single plasmid. Any one of the many methods of transfection, such as the calcium phosphate-DNA coprecipitation method, known to those skilled in the art may be used, as long as the method is compatible with the cell line chosen. The second step of the method involves determining the level of mRNA target transcripts in the cell relative to the level of mRNA transcript produced in a cell by a vector encoding only mRNA sequence. The amount of mRNA cleavage correlates directly with the efficacy of a ribozyme construct *in vivo*. A working example of this method is outlined in Example 5.

Additionally, the method described above may also be used to distinguish between ribozyme effects and antisense effects. This may be accomplished by employing mutant versions of the ribozyme that retain base pairing activity with targeted mRNA but lack enzymatic activity. The cell culture assessment of both ribozyme efficacy and antisense effect is an important prerequisite for the effective application of each of the invention methods for in vivo control of gene expression. Finally, the cell culture method described herein may also be used in a very similar manner to determine the efficacy of an antisense oligonucleotide for controlling gene expression.

The present invention is further illustrated by the following examples (see below). Neuregulin mRNA was a target for inactivation, since the recent demonstration of the inactivation of the genes for neuregulin and its receptors in the mouse provided a basis for comparison with ribozyme-generated phenotypes. Guidelines were developed for ribozyme target site selection that are generally applicable to any mRNA sequence (Example 1), and then used to obtain several ribozymes capable of cleaving chicken neuregulin mRNA (Example 2). The ribozyme sequences were embedded within the anticodon loop of a tRNA (Example 3) and tested for efficacy in the cell culture assay (Example 5). Production of a retrovirus carrying a neuregulin-cleaving tRNA-ribozyme expression vector is described in Example 6. Introduction of the retrovirus into blastoderm-stage embryos produced a lethal embryonic phenotype that resulted from failure of ventricular trabeculation in the developing heart, a copy of the phenotype of the mouse NRG mutations (Example 7). Additionally, the localized delivery of the neuregulin-cleaving ribozyme to the developing retina was found to inhibit both the differentiation of retinal ganglion cell neurons and the proliferation of the neuroepithelial cells from which they derive (Example 8). The tRNA-ribozyme technology disclosed herein provides a simple, effective, and easily adaptable method of conditional gene inactivation in vertebrates.

The following specific Examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims.

Example 1. Guidelines for Ribozyme Target Site Selection.

In order to establish rational rules for ribozyme cleavage site selection, an extensive literature survey was conducted for published data directed to the efficacy of multiple antisense reagents (mostly ribozymes or anti-sense oligonucleotides) directed against a single target RNA under physiological conditions. Results from test tube reactions with small substrate RNAs were excluded from the survey. The RNA secondary structure prediction program MFOLD developed by Zuker and colleagues (Jaeger et al, 1989, *Proc. Natl. Acad. Sci.*, 86:7706-710; Zuker, 1989, *Science*, 244:48-52) and widely available on the University of Wisconsin Genetics Computer Group (GCG) software package, was used to predict the minimum free energy or "optimal" secondary structures of each of the target RNAs. Importantly, the program was also used to predict 3-5 near-minimum free energy ("sub-optimal") structures for these targets. Finally, the predicted structures were compared with the observed efficacy of the antisense reagents, as measured by reduction in the level of target RNA or the level or bioactivity of the corresponding encoded protein.

The following table represents the analysis of 128 pairs of predicted minimum and near-minimum free energy secondary structures of target RNAs and their corresponding antisense reagents, in 22 different large RNAs (see Table 1).

Table 1. Computational analysis of target site accessibility.

Target RNA	Reagent[a]	Sites tested[b]	Correlated sites[c]	Reference
drosophila ftz	RBZ	1	1	Zhao & Pick, 1993
bovine α-lactalbumin	RBZ	5	4(1)	L'Huillier et al., 1992
bovine α-lactalbumin	RBZ	1	1	L'Huillier et al., 1992
human growth hormone	RBZ	7	7	Lieber & Strauss, 1995
drosophia paired	RBZ	2	2	Vanario-Alonso, 1995
human mdr1	RBZ	1	1	Holm et al., 1995
rabbit stromelysin	RBZ	4	2(2)	Flory et al., 1996
mouse glucokinase	RBZ	1	1	Efrat et al., 1994
human pleiotrophin	RBZ	2	2	Czubayko et al., 1994
Tn9 CAT	RBZ	3	2	Cameron & Jennings, 1989
rat Pit-1	RBZ	5	2(2)	Bertrand et al., 1994
chick neuregulin 1	RBZ	4	3	Example 4
human mdr1	RNase H	10	7(3)	Ho et al., 1996
chick islet-1	ASO	2	2	Pfaff et al., 1996
chick Slug	ASO	2	2	Nieto et al., 1994
chick dHAND	ASO	2	2	Srivastava et al., 1995
chick eHAND	ASO	2	2	Srivastava et al., 1995
mouse SNAP-25	ASO	2	2	Osen-Sand et al., 1993
hepatitis C virus	ASO	19	13(4)	Wakita & Wands, 1994
human PKC-α	ASO	20	11(5)	Dean et al., 1994
human E-selectin	ASO	18	12(1)	Bennett et al., 1994
human VCAM-1	ASO	15	11(1)	Bennett et al., 1994

[a] RBZ, hammerhead ribozyme; ASO, antisense oligonucleotide. [b] Number of target sites tested experimentally for reduction in target RNA or protein or bioactivity. [c] Number of target sites within a computer-predicted RNA secondary structure that: (1) conform to Rule 2 and one or more remaining rules (below); and in addition (2) result in a measured reduction in target RNA or protein or bioactivity of at least 30%. Numbers in parentheses indicate sites predicted to have

poor accessibility for RBZs or ASOs that have also been demonstrated to exhibit low inhibitory activity (<30% reduction in target RNA, protein, or bioactivity levels) in experimental assays.

The correlation between the experimentally determined efficacy of the antisense reagents and computer predictions of the accessibility of their targets for base-pairing is stronger in the case of ribozymes than antisense oligonucleotides. This is not surprising, since the mechanism of ribozyme action necessarily depends on the accessibility of base-pairable target regions, while the action of antisense oligonucleotides is also a function of oligonucleotide backbone modifications, interference with protein synthesis, and alteration of RNA processing.

Based on the above analysis, a set of rules was formulated for the rational design of ribozyme or antisense segments that are predicted to reliably base-pair with target RNAs. These rules apply mainly to the selection of a cleavage site and the flanking sequences that will base pair with the arms of hammerhead ribozymes, but many are also applicable to other types of ribozymes and antisense oligonucleotides. The rules require the prediction of both the optimal structure, as well as 3 to 5 sub-optimal structures that are within 2% of the computed minimum free energy of the target RNAs. This requirement derives from the fact that the reliability of computer prediction for very large mRNAs is still not high, and a predicted target structure (a stem loop, for example) must therefore appear in both minimum and more than one near-minimum free energy prediction in order to be informative with respect to the rules below. Although this requirement is computationally intensive for RNAs approaching 1,400 nucleotides in length (the MFOLD limitation in the GCG package we used), it is much less onerous if the program is run on a workstation or a supercomputer.

The guidelines for ribozyme target site selection established by our studies are as follows:

1. Target sequences predicted to be embedded within a long base-paired stem (double-stranded region) are to be avoided. For reliable design, these

unfavored regions must be predicted to appear in both minimum and near-minimum free energy structures.

2. For hammerhead ribozymes, the selected cleavage site should fall within a loop that is not smaller than four nucleotides. Again, reliable design requires that this loop appear in both minimum and near-minimum free energy structures. A loop in the target is essential for the formation of the "kissing complex" through which many natural antisense RNAs first base pair with their targets. However, a predicted large loop (>25 nt) should be avoided, since it may be involved in the formation of a tertiary RNA structure.

3. One, and preferably both, of the 5' and 3' ends of the antisense segment should fall within a single-stranded rather than a stem region. The "kissing complexes" of natural antisense-target interactions in prokaryotes are typically resolved and extended through base-pairing of a free 5' end of the antisense RNA, and the survey of published results indicates that antisense reagents whose 5' and 3' ends are predicted to hybridize to non-base-paired regions are more consistently effective.

4. Target sequences within the first several hundred nucleotides of a large RNA molecule are favored. The predicted secondary structures in this region are more likely to form in a nascent mRNA molecule and are less likely to be affected by the structure of the 3' region of the mRNA. It is best to include a maximal length (e.g. 1400 nucleotides) of 5' end sequence of a target RNA for modeling.

5. Short antisense segments (7-12 nucleotides for each arm of a hammerhead ribozyme, 12-23 nucleotides for an antisense oligo) are better than longer ones. Long antisense segments that have a significant propensity to form stable secondary structures on their own, especially within sequence designed to base-pair with a predicted loop within the target, are to be avoided.

Example 2. Design of Neuregulin-Cleaving Ribozymes

The above-described rules for ribozyme target site selection (Example 1) were used to guide the design of ribozymes capable of inhibiting neuregulin (NRG) expression in vivo (Fig. 1). Based on the cDNA sequence of chicken NRG (previously designated ARIA) (Falls et al., 1993, *Cell* 72:801-815), four GUC/A target sites - at RNA nucleotides 89, 156, 438, and 637 - were chosen without prior knowledge of their position within predicted minimal free energy structures. The MFOLD computer program was used to produce an optimal and four suboptimal free energy structures of the first 1400 nucleotides of the NRG mRNA. It was determined that the RZ_{NRG156} (SEQ ID NO:2) cleavage site and its flanking sequences are largely unfavorable in that they are predicted to be buried within a double-stranded region of the optimal structure, and within the same region in three out of four suboptimal structures. Similarly, the RZ_{NRG637} (SEQ ID NO:4) cleavage site was only accessible in an internal loop of the optimal structure, and in only one of the sub-optimal structures; its 3' flanking arm is predicted to be double-stranded in these two structures. In contrast, the RZ_{NRG89} (SEQ ID NO:1) cleavage site is located within a favored, multibranched loop that appears in the optimal and in three of the sub-optimal structures, although one of its flanking arms encounters a stable stem in these structures. Similarly, the cleavage site for RZ_{NRG438} (SEQ ID NO:3) is located within a relatively favored internal loop of the optimal and in three of the sub-optimal energy structures; in addition, both of the arms flanking this site, together with their ends, land within favored single-stranded or loose stem regions in these predicted structures. Based on these structural considerations and the rules outlined above, the predicted order of efficacy for the four ribozymes is $RZ_{NRG438} > RZ_{NRG89} > RZ_{NRG637} > RZ_{NRG156}$.

The set of four designed ribozymes (RZ_{NRG438}, RZ_{NRG89}, RZ_{NRG637}, and RZ_{NRG156}) was predicted to target all of the bioactive isoforms of neuregulin mRNA. Specificity was set by the inclusion of 9-12 flanking nucleotides on both

sides of the catalytic domain of the enzyme, which are complementary to sequences flanking the target sites in the NRG mRNA.

Example 3. Ribozyme-tRNA Chimeras.

Ribozyme sequences were embedded within the anticodon loop region of a synthetic chicken alanine tRNA gene and the targeting ribozyme was expressed as a ribozyme-tRNA hybrid (Fig. 1). All DNA templates encoding NRG ribozymes were synthesized as complementary oligonucleotides, which were annealed to generate *Pstl* sticky ends, and then inserted into an engineered *Nsil* site in the anticodon of a synthesized tRNA gene based on a chicken alanine tRNA sequence (Mezquita and Mezquita, 1992, *Nucl. Acids Res.*, 20:5477).

Example 4. Catalytically Inactive Mutant Ribozyme Controls for Simple Antisense Action.

Mutant ribozymes were also developed. These ribozymes were utilized to distinguish between effects due to catalytic cleavage versus effects due to simple antisense action. We engineered a single base change (A to G) in the catalytic core of all four anti-neuregulin ribozymes. The mutant ribozyme sequences were as follows (with the mutated G shown underlined):

mRZ_{NRG89} (SEQ ID NO:5):

5'GTAACTCTGTTCTGATGAGTCCGAGAGGACGAGACGTCCGTCTGAGCTCTGCA-3'

mRZ_{NRG156} (SEQ ID NO:6):

5'-GCACTAGCTTCTCTGATGAGTCCGAGAGGACGAGACCCACAGCAAGAGCTCTGCA-3'

mRZ_{NRG438} (SEQ ID NO:7):

5'-GTTGTGAGATCTGATGAGTCCGAGAGGACGAGACTTGTCCCAGAGCTCTGCA-3'

mRZ_{NRG637} (SEQ ID NO:8):

5'-GCCGTTTCTGGCTGATGAGTCCGAGAGGACGAGACAGTTCCTCGAGCTGCA-3'

This point mutation in the ribozyme abolishes most cleavage activity but maintains the conformation, stability, and antisense sequence of the flanking arms of the hammerhead ribozymes (Ruffner et al., 1990, *Biochemistry*, 29:10695-10702). The point mutation in the catalytic domain of the ribozymes was introduced by PCR methods. The structures of all normal and mutated ribozyme-tRNA transgenes were confirmed by sequencing.

Example 5. Cell Culture Test of Ribozyme Efficacy

A cell culture transfection assay was set up to measure the activity of the normal and mutant ribozymes in human embryonic kidney (293T) cells. The ctRNA expression cassette containing either a normal ribozyme, a mutant ribozyme, or no ribozyme, was sub-cloned into a NRG expression vector (p12.7) to generate a single plasmid dual-expression vector (Fig. 2A). The backbone of the vector was pCDNAI/Amp (available from Invitrogen).

Approximately 10^6 human embryonic kidney 293T cells were seeded onto 60 mm dishes 1 day before transfection. Each dish was transfected with 1 μg of the reporter plasmid pEGFPC1 and 6 μg of the dual-expression plasmid by a polyethylenimine-mediated DNA transfection method (Boussif et al., 1995, *Proc. Natl. Acad. Sci.*, 92:7297-7301). Approximately 20 hours later, cells were lysed in TRI reagent (Molecular Research Center, Inc.) and total RNAs were subsequently isolated and analyzed by northern blot. Signals were recorded using a model 400

Molecular Dynamics phosphorimager. Every 293T cell transfected with the plasmid carries a fixed 1:1 copy ratio of ribozyme to target gene. (The actual ratio of ribozyme RNA to target mRNA is a function of the relative strength of the pol III-mediated promoter of the ribozyme-tRNA gene and the pol II-mediated cytomegalovirus (CMV) promoter driving the target NRG gene.) Transcription of the ribozyme-tRNA cassette was oriented in the opposite direction from NRG-1 gene transcription, to exclude cis-acting ribozyme effects and ensure only trans-acting ribozyme effects would be observed. The transfection efficiency was measured by co-transfecting pEGFPC1, a green fluorescent protein (GFP) reporter plasmid (from Clontech).

Total RNAs were isolated from transiently transfected human embryonic kidney 293T cells and fractionated on an 1% agarose denaturing gel. After transfering onto nylon membrane, blots were hybridized with probes against neuregulin RNA or green fluorescent protein RNA. Radioactivity in each band was quantitated on a phosphorimager, the intensity of the neuregulin RNA signal relative to that of GFP RNA was determined, and this ratio was then plotted relative to the ratio obtained for the NRG-ctRNA cassette without ribozyme, whose relative signal was set to 100%.

Northern analysis of total RNA isolated from transiently transfected cells demonstrated a pronounced reduction of neuregulin mRNA levels relative to control (tRNA alone), from approximately 45% relative to control for RZ_{NRG156}, to approximately 5% relative to control for RZ_{NRG438}; in each case, reduced neuregulin mRNA expression was largely due to the catalytic activity of the ribozyme. Partial antisense inhibitory effects were observed for the mRZ_{NRG156} and mRZ_{NRG438} A to G mutants, but their catalytically active counterparts were always superior in effect. For RZ_{NRG89} and RZ_{NRG637}, all of the measured reduction in neuregulin mRNA levels was due to ribozyme rather than antisense action.

Since the predicted order of efficacy for the four designed neuregulin-cleaving ribozymes was $RZ_{NRG438} > RZ_{NRG89} > RZ_{NRG637} > RZ_{NRG156}$, the theoretical

ordering of potency corresponded well to the observed ability of each ribozyme to reduce target neuregulin mRNA levels. Expression of RZ_{NRG156}, RZ_{NRG637}, RZ_{NRG89}, and RZ_{NRG438} resulted in neuregulin mRNA levels that were -50%, -35%, -20%, and -5% relative to control, respectively.

Example 6. Construction of a Retroviral Expression Vector Encoding Ribozyme-tRNA Hybrid Molecules.

As an *in vivo* delivery vehicle, an avian replication-competent retroviral vector was used into which the tandemly arrayed ribozyme-tRNA genes were inserted to generate RCAS/$4XRZ_{NRG}$ (Fig. 2B). Ribozyme-tRNA transgenes linked in tandem are transcribed as individual RNA transcripts due to the presence of strong transcription termination signals at the 3' end of each transgene, which signals also prevent transcription of the non-coding strand of the retroviral genes.

In order to construct RCAS/$4XRZ_{NRG}$, (Fig. 2B) four ribozyme-tRNA minigenes (each ~170 bp in length) were clustered together in the same orientation, and then subcloned into the Cla 12 adaptor vector (Hughes et al, 1987, *Journal of Virology*, 61:3004-3012). The use of this adaptor plasmid allows the conversion of the cluster of ribozyme-tRNA minigenes into a single *ClaI* fragment, which is then suitable for insertion into the *ClaI* site of the retroviral vector RCASBP(A) (Petropoulos and Hughes, 1991, *Journal of Virology*, 65: 3728-3737), downstream of the *env* gene and in opposite orientation to transcription driven by the left retroviral LTR promoter. Similarly, a *ClaI* fragment containing the RZ_{NRG156}-tRNA transgene and the human placental alkaline phosphatase coding sequence (used as a marker) was cloned into the same viral vector to generate RCAS/RZ_{NRG156}. High titer (~109 infectious units/ml) retroviral stocks were prepared in chick embryonic fibroblasts by standard methods (Morgan and Fekete, 1996, in *Methods in Avian Embryology*, p.185-218).

Example 7. Inhibition of Neuregulin Expression in Chicken Embryos.

In both the mouse and the early chick embryo, the heart is a major site of neuregulin expression. To determine whether ribozymes could antagonize neuregulin function during chick heart development RCAS/4XRZ$_{NRG}$ was injected at the blastoderm stage (approximately 4 hr incubation). A retrovirus carrying RZ$_{NRG156}$ which only reduced NRG mRNA to approximately 45% in the 293T cell transfection assay (Example 4) was also prepared and injected into chicken embryos at the blastoderm stage. This RCAS/AP/RZ$_{NRG156}$ virus was used as a "heterozygous knock-out" control for any non-specific effects on development that might be due to retroviral infection and replication alone, or to the expression of an aberrant tRNA.

For virus infection, specific pathogen free White Leghorn eggs (SPAFAS, Inc.) were used. Normally, eggs were placed on their sides except for the blastoderm stage injection, when eggs were placed in standing position with the air sac on top, incubated at 38°C in a high-humidity incubator, and windowed before injection. Injection was performed as described (Morgan and Fekete, 1996, in *Methods in Avian Embryology*, p.185-218).

The injection of retrovirus into the early embryo resulted in the infection of the majority of blastoderm cells and their progeny, as visualized with primary antibodies directed against viral gag proteins. At 44 hr postinfection, prior to cardiac trabeculation in the chick, both RCAS/4XRZ$_{NRG}$ infected embryos and control virus infected embryos had developed normally. After a further 24 hr, however, all embryos infected with RCAS/4XRZ$_{NRG}$ (n=16) were found to lack the normal network of yolk sac blood vessels, while this network of vessels was clearly present in 17 out of 23 RCAS/AP/RZ$_{NRG156}$ infected embryos. At 90 hr postinjection, an even more dramatic difference was observed, in that there was no observable heartbeat or blood flow in most RCAS/4XRZ$_{NRG}$ infected embryos, which were also markedly deformed. Since this time is equivalent in terms of cardiac development to the embryonic age at which the mouse *NRG-1* mutants die,

histological sections of hearts from RCAS/4XRZ$_{NRG}$ and control embryos were analyzed. Myocardial trabeculae were missing from the ventricles of embryos infected with the RCAS/4XRZ$_{NRG}$ virus, whereas ventricles from control injections were normally trabeculated, even though these control ventricles were extensively infected with RCAS/RZ$_{NRG156}$. The defect in myocardial trabeculation that we observed in these chick embryo experiments is very similar in histology, time of onset, and extent to the cardiac muscle defect seen in mice lacking either NRG or its receptor (Meyer and Birchmeier, 1995, *Nature* 378:386-390; Lee et al., *Nature* 378:386-390; Gassmann et al., *Nature* 378: 390-394). In the mouse, this defect results from the loss of NRG signaling, delivered from the endocardial endothelium to the myocardial muscle. Consistent with this signaling pathway, we found that NRG was barely detectable inside hearts infected by RCAS/4XRZ$_{NRG}$, but that the expression level of the ErbB2 and ErbB4 receptors was unaffected (data not shown). We used a chick muscle specific marker 13F4 to demonstrate that myocardial wall development occurs normally in the RCAS/4XRZ$_{NRG}$-infected heart, an effect that also replicates the cardiac histology of the mouse *NRG*, *ErbB2*, and *ErbB4* gene mutants.

The *in vivo* expression of ribozyme-tRNA transgenes was confirmed by northern blot. Total RNA from untransfected chick embryonic fibroblast cells (CEF), CEF transiently transfected with RCAS/RZ$_{NRG156}$ plasmid DNA, CEF transfected with RCAS/4XRZ$_{NRG}$ plasmid DNA, and uninfected E7.5 chick eye tissue, were fractioned on an 2.5% agarose denaturing gel. After transfer to nylon membrane, the blot was hybridized with riboprobes containing RZ$_{NRG89}$, RZ$_{NRG156}$, and RZ$_{NRG637}$ antisense sequences. The probes detected both cellular tRNA transcripts and ribozyme-tRNA hybrids. The hybrids appeared as a single band in both the single hybrid and four hybrid transfections, indicating that the four ribozyme-tRNA transgenes in RCAS/4XRZ$_{NRG}$ were independently transcribed.

Example 8. Inhibition of Neuregulin Expression in Chick Retina.

The ability of invention ribozyme-tRNA hybrids to inhibit expression of neuregulin in chick retina was also investigated. Based on the embryonic expression patterns of NRG and its ErbB receptors, it has been postulated that these molecules are regulators of neurogenesis and/or neuronal differentiation in the developing central nervous system. In chick retina, NRG is expressed at the earliest (optic stalk and optic cup) stages of development and recent cell culture experiments have suggested an important role for NRG in the development of retinal neurons (Bermingham-McDonogh et al., 1996, *Development*, 122:1427-1438). At the onset of retinal histogenesis, the optic cup is composed of undifferentiated stem cells, and the first cells to exit the cell cycle and differentiate are retinal ganglion cell (RGC) neurons.

To demonstrate the ability to shut off neuregulin expression in the retinal ganglion cell neurons, the RCAS/4XRZ$_{NRG}$ or RCAS/RZ$_{NRG156}$ virus was injected into the optic cup at stage 13-14 (E2) and the injection repeated once 12 hours later. Embryos were then incubated for an additional 96 hours. Retinal sections were stained with anti-gag antibodies to identify infected cells, and then adjacent sections double-stained with the NRG antibody and the monoclonal antibody RA4, which recognizes an RGC-specific antigen shortly after these cells are born. By immunostaining, the percentage of Gag (p27) positive cells was determined to be 58.6%±15.1 in RCAS/RZ$_{NRG156}$ infected retina and 62.9%±7.6 in RCAS/4XRZ$_{NRG}$ infected retina.

In chick retinal regions infected by RCAS/4XRZ$_{NRG}$ virus, the number of NRG positive cells was substantially reduced, compared to RCAS/RZ$_{NRG156}$ - infected retinal regions. It has previously been shown that cells positive for RA4 are confined to the inner surface of the central retina, within the population of developing RGC neurons (McLoon and Barnes, 1989, *Neuroscience*, 9:1424-1432). At E7, this population comprises multiple cell layers with strong RA4 expression in the central retina of RCAS/RZ$_{NRG156}$ infected eyes. Weak RA4 expression and a

reduced number of cell layers in RCAS/4XRZ$_{NRG}$ virus infected retina were observed. To obtain a quantitative assessment of NRG effects on RGC differentiation, dissociated E5 retinal cells were stained with a monoclonal antibody against the transcription factor Islet-1, which marks all RGC nuclei at this stage of retinal development (Austin et al., 1995, *Development*, 121:3637-3650). The percentage of Islet-1 positive cells was reduced from 13.0%±3.6 in RCAS/RZ$_{NRG156}$ infected retina, which is very similar to previous observations on uninfected retina, to 4.9%±1.2 in RCAS/4XRZ$_{NRG}$ virus infected retina.

Since NRG appears to act both as a differentiation agent and a mitogen for many developing cells, it was reasoned that it might also stimulate division of retinal stem cells. To test this possibility, dissected retinae were cultured in the presence of 1 mM BrdU, which is incorporated into the newly synthesized DNA of dividing cells. After a 2 hour labeling period in 1mM BrdU prior to trypsin treatment (Altshuler and Cepko, 1992, *Development*, 114:947-957), E5 retinal cells were dissociated as described (Austin et al., 1995, *Development*, 121:3637-3650) and the number of BrdU$^+$ cells was compared between injected RCAS/4XRZ$_{NRG}$ and control RCAS/RZ$_{NRG156}$ samples. A substantial reduction in mitotic activity in RCAS/4XRZ$_{NRG}$-infected retinae (BrdU$^+$ cells: 2.3 ± 1.0%)compared to RCAS/RZ$_{NRG156}$-infected retinae (BrdU$^+$ cells: 8.6 ± 3.1%) was observed.

All documents cited in the above specification are herein incorporated by reference. Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

What is claimed is:

1. A retroviral expression vector comprising nucleic acid encoding at least one ribozyme, wherein said ribozyme specifically cleaves a non-viral mRNA.

2. An expression vector of Claim 1, wherein the ribozyme comprises a ribozyme-tRNA hybrid.

3. An expression vector of Claim 2, wherein the ribozyme is positioned in the anticodon region of the anticodon loop of the tRNA.

4. An expression vector of Claim 1, wherein the ribozyme is characterized as having a motif characteristic of a hammerhead ribozyme, a group I intron, a hairpin ribozyme, VS RNA, the hepatitis Delta virus, or RnaseP RNA.

5. An expression vector of Claim 1, wherein said mRNA encodes a neuregulin, GDF-8, or an interferon.

6. A viral expression vector comprising nucleic acid encoding at least one ribozyme-tRNA hybrid, wherein the ribozyme component of said hybrid specifically cleaves a non-viral mRNA and wherein said expression vector is in association with a delivery vehicle selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a lentivirus, a herpes simplex virus, and a vaccinia virus.

7. A viral expression vector of Claim 6, wherein the ribozyme component of said hybrid is characterized as having a motif characteristic of a hammerhead ribozyme, a group I intron, a hairpin ribozyme, vs RNA, the hepatitis Delta virus, or RnaseP RNA.

8. A viral expression vector of Claim 6, wherein said mRNA encodes a neuregulin, GDF-8, or an interferon.

9. A host cell comprising an expression vector of Claim 1.

10. A host cell comprising an expression vector of Claim 6.

11. A non-human transgenic vertebrate animal comprising cells modified with an exogenous nucleic acid encoding at least one ribozyme, wherein said ribozyme specifically cleaves an mRNA endogenous to said transgenic animal.

12. A transgenic vertebrate animal of Claim 11, wherein said vertebrate animal is selected from the group consisting of murine, porcine, bovine, ovine, and piscine animals.

13. A transgenic vertebrate animal of Claim 11, wherein said vertebrate animal is avian.

14. A transgenic vertebrate animal of Claim 13, wherein said vertebrate animal is a chicken or a turkey.

15. A non-human transgenic animal of Claim 11 which is an embryo comprising cells modified with an exogenous nucleic acid which encodes at least one ribozyme, wherein said ribozyme specifically cleaves an mRNA endogenous to said embryo.

16. A transgenic vertebrate embryo of Claim 15, wherein said embryo is selected from the group consisting of avian, murine, porcine, bovine, ovine, and piscine embryos.

17. A method of controlling expression of a gene in an animal, wherein said method comprises:

(a) introduction of an expression vector of Claim 1 into said animal;

(b) transcription of the ribozyme sequence contained in the expression vector; and

(c) cleavage by the transcribed ribozyme of about 5% to about 100% of mRNA transcripts of the target gene.

18. A method of claim 17 wherein the animal is in the embryonic stage.

19. A method of Claim 17, wherein said animal is selected from the group consisting of avian, bovine, human, murine, ovine, porcine, and piscine animals.

20. A method of modifying embryonic development, wherein said method comprises:

(a) introduction of an expression vector of Claim 1 into an embryo at a desired developmental stage;

(b) transcription of the ribozyme sequence contained in said expression vector; and

(c) cleavage by the transcribed ribozyme of about 5% to about 100% of mRNA transcripts of a gene in said cell.

21. A method of Claim 20, wherein said embryo is selected from the group consisting of avian, bovine, human, murine, ovine, porcine, and piscine embryos

22. A recombinant ribozyme molecule which specifically cleaves neuregulin mRNA, GDF-8 mRNA, or interferon mRNA.

23. A ribozyme molecule of Claim 22 which comprises at least 14 contiguous nucleotides complementary to said mRNA.

24. A ribozyme molecule of Claim 22, wherein said ribozyme molecule has a motif characteristic of a hammerhead ribozyme, a group I intron, a hairpin ribozyme, VS RNA, the hepatitis Delta virus, or RnaseP RNA.

25. A method of controlling expression of a gene in an animal, wherein said method comprises:

(a) introduction of a ribozyme molecule of Claim 22 into said animal; and

(b) ribozyme-facilitated cleavage of about 5% to about 100% of the mRNA transcripts of the target gene.

26. A method of Claim 25, wherein said animal is selected from the group consisting of avian, bovine, human, murine, ovine, porcine, and piscine animals.

27. A method of controlling expression of a gene in an embryo, wherein said method comprises:

(a) introduction of a ribozyme molecule of Claim 22 into said embryo; and

(b) ribozyme-facilitated cleavage of about 5% to about 100% of the mRNA transcripts of the target gene.

28. A method of Claim 27, wherein said embryo is selected from the group consisting of avian, murine, porcine, bovine, ovine, and piscine embryos.

29. A host cell comprising a ribozyme molecule of Claim 22.

30. A method of determining the efficacy of a ribozyme sequence for controlling gene expression, wherein said method comprises:

(a) transfecting a cell in vitro with an expression vector comprising nucleic acid encoding both a ribozyme and an mRNA sequence, wherein said ribozyme is capable of cleaving said mRNA sequence; and

(b) determining the level of mRNA transcripts in said cell relative to the level of mRNA transcripts produced in a cell by a vector encoding only the mRNA target sequence.

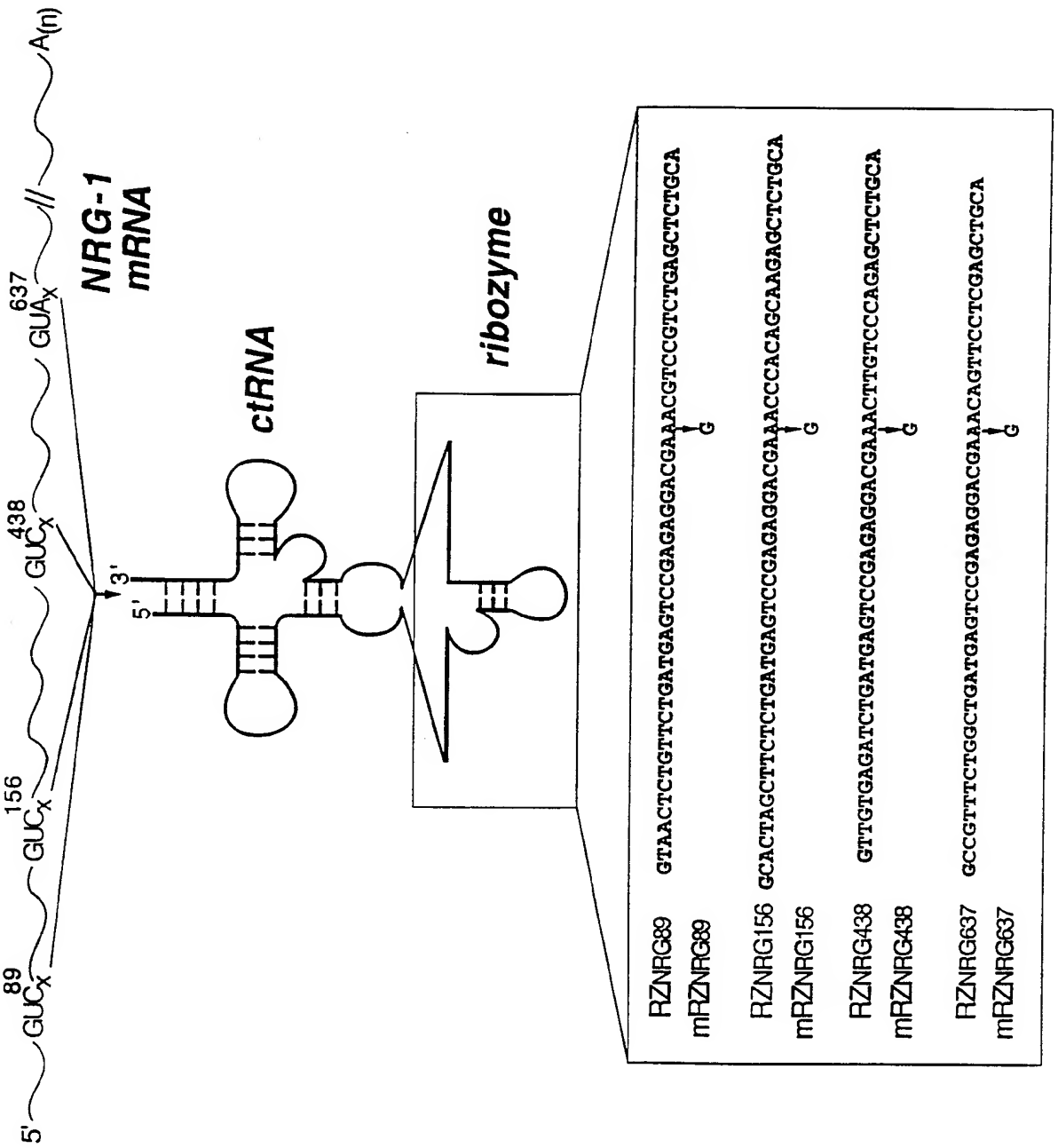
5'
GUC
89
GUC
156
GUC
438
GUA
637
A(n)
NRG-1
mRNA
ctRNA
ribozyme
RZNRG89 GTAACTCTGTTCTGATGAGTCCGAGAGGACGAAACGTCCGTCTGAGCTCTGCA
mRZNRG89
RZNRG156 GCACTAGCTTCTCTGATGAGTCCGAGAGGACGAAACCCACAGCAAGAGCTCTGCA
mRZNRG156
RZNRG438 GTTGTGAGATCTGATGAGTCCGAGAGGACGAAACTTGTCCCAGAGCTCTGCA
mRZNRG438
RZNRG637 GCCGTTTCTGGCTGATGAGTCCGAGAGGACGAAACAGTTCCTCGAGCTGCA
mRZNRG637

Fig. 1

2A


RZ
PCMV
NRG ORF/pA
ctRNA

2B

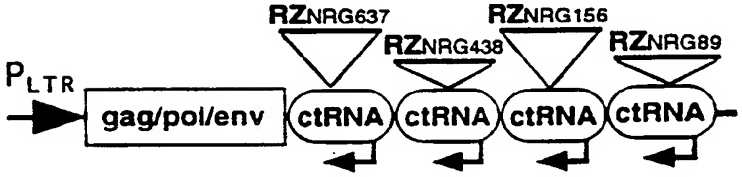
RZNRG637
RZNRG438
RZNRG156
RZNRG89
PLTR
gag/pol/env
ctRNA
ctRNA
ctRNA
ctRNA

Fig. 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: RIBOZYME-MEDIATED CONTROL OF GENE EXPRESSION

(iii) NUMBER OF SEQUENCES: 8

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0. Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
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(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTAACTCTGT TCTGATGAGT CCGAGAGGAC GAAACGTCCG TCTGAGCTCT GCA 53

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACTAGCTT CTCTGATGAG TCCGAGAGGA CGAAACCCAC AGCAAGAGCT CTGCA 55

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTGTGAGAT CTGATGAGTC CGAGAGGACG AAACTTGTCC CAGAGCTCTG CA 52

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCGTTTCTG GCTGATGAGT CCGAGAGGAC GAAACAGTTC CTCGAGCTGC A 51

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTAACTCTGT TCTGATGAGT CCGAGAGGAC GAGACGTCCG TCTGAGCTCT GCA 53

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCACTAGCTT CTCTGATGAG TCCGAGAGGA CGAGACCCAC AGCAAGAGCT CTGCA 55

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTGTGAGAT CTGATGAGTC CGAGAGGACG AGACTTGTCC CAGAGCTCTG CA 52

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCGTTTCTG GCTGATGAGT CCGAGAGGAC GAGACAGTTC CTCGAGCTGC A 51